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Identification of Anaerobic Bacteria by Bruker Biotyper Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry with On-Plate Formic Acid Preparation

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Identification of anaerobic bacteria using phenotypic methods is often time-consuming; methods such as 16S rRNA gene sequencing are costly and may not be readily available. We evaluated 253 clinical isolates of anaerobic bacteria using the Bruker MALDI Biotyper (Bruker Daltonics, Billerica, MA) matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) system with a user-supplemented database and an on-plate formic acid-based preparation method and compared results to those of conventional identification using biochemical testing or 16S rRNA gene sequencing. A total of 179 (70.8%) and 232 (91.7%) isolates were correctly identified to the species and genus levels, respectively, using manufacturer-recommended score cutoffs. MALDI-TOF MS offers a rapid, inexpensive method for identification of anaerobic bacteria.

Identification of anaerobic bacteria using phenotypic methods is time-consuming and may produce inconclusive results, leading to application of costly methods, such as 16S rRNA gene sequencing. Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) has been used predominantly for aerobic bacterial identification and is now in use in many clinical laboratories (1–5). A small number of studies have preliminarily evaluated this technology for identification of anaerobic bacteria (4, 6–12), but this application is not in widespread clinical use.

We showed that off-plate protein extraction yields increased scores and, consequently, a higher percentage of isolates identified compared to direct on-plate testing of colonies for *Corynebacterium* species and Gram-positive cocci using the Bruker Biotyper system (Bruker Daltonics, Billerica, MA) (1, 13). Others have applied this strategy to anaerobic bacteria (6, 14, 15). In our experience, off-plate extraction is cumbersome for laboratory technologists and results in waste production and high cost. We recently showed that an easier-to-use on-plate testing method using 70% formic acid demonstrated results comparable to those of off-plate extraction for *Corynebacterium* species and yeast (16). Using the Bruker Biotyper system, Justesen and coworkers performed a similar on-plate preparation technique using 1 μl of 70% formic acid for anaerobic bacteria which were not identified using direct on-plate testing without an extraction step (7). Unpublished studies conducted by our group have shown that on-plate formic acid testing yields results equivalent to those of off-plate extraction for staphylococci, streptococci, and aerobic and facultatively anaerobic Gram-negative bacilli. Universal application of this method for identification of bacterial colonies is potentially fast and cost-effective and allows application of MALDI-TOF MS without antecedent Gram staining.

We evaluated the Bruker MALDI Biotyper for identification of clinically isolated anaerobic bacteria using on-plate formic acid preparation.

MATERIALS AND METHODS

Bacterial isolates. A total of 253 clinical isolates of anaerobic bacteria were obtained from the Mayo Clinic Bacteriology Laboratory over a 6-month period. All were initially isolated on CDC anaerobic blood agar plates (BD Diagnostic Systems, Sparks, MD) incubated in an anaerobic chamber (Coy anaerobic glove box; Coy Laboratory Products, Grass Lake, MI) at 35°C. *Propionibacterium acnes* was identified using catalase and quick indole testing, with all other identification performed by sequencing of the first 500 bases of the 16S rRNA gene as described previously (17), followed by sequence analysis using MicroSeq (MicroSeq ID, version 2.0, AB_bacterial500LIB_2.2; Applied Biosystems, Carlsbad, CA) and the Mayo Clinic custom anaerobe library (J. E. Rosenblatt, presented at Anaerobe 2004, the 7th Biennial Congress of the Anaerobe Society of the Americas, Annapolis, MD, 19 to 21 July 2004). RipSeq Mixed software (Isentio, Sunnyvale, CA) was used if an acceptable match (>99.0%) was not identified or if the consensus sequence had fewer than 420 bases. To reduce user variance, MALDI-TOF MS was performed by a single individual on isolates subcultured to CDC anaerobic blood agar plates following routine identification and grown for 48 to 72 h in anaerobic jars containing an anaerobic atmosphere generator pack (AnaeroPack; Remel, Lenexa, KS) at 35°C.

MALDI-TOF mass spectrometry. Isolates were analyzed using a formic acid-based direct, on-plate preparation method. One microliter of 70% formic acid (Fluka; Sigma-Aldrich, St. Louis, MO) per well was deposited onto the MALDI-TOF MS steel anchor plate (BigAnchor 96-well plate; Bruker Daltonics). Colonies were smeared into the formic acid and allowed to dry. The dried mixture was overlain with 2 μl of matrix solution (α-cyano-4-hydroxycinnamic acid [HCCA]; Bruker Daltonics) dis-

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solved in 50% acetonitrile, 47.5% water, and 2.5% trifluoroacetic acid (Fluka; Sigma-Aldrich, St. Louis, MO) and allowed to dry prior to analysis using the MALDI Biotyper. A bacterial test standard (BTS; Bruker Daltonics) was used for instrument calibration. A positive control (*Staphylococcus aureus* ATCC 25923) and a negative control (formic acid and matrix) were included with each run.

A MicroFlex LT mass spectrometer (Bruker Daltonics) was used for analysis. Spectra were analyzed using the Bruker Biotyper 3.0 software and library version 3.3.1.0 (4,613 entries), supplemented with mass spectra from an in-house collection of 87 anaerobic isolates encompassing 39 species (see Table S1 in the supplemental material). Manufacturer-recommended cutoff scores were used for identification, with scores of ≥2.000 indicating identification to the species level, scores between 1.700 and 1.999 indicating identification to the genus level, and scores of <1.700 indicating no identification. Isolates producing scores of <1.700 were retested once with the highest score used for final analysis. 16S rRNA gene sequencing was considered the reference method for discrepant results.

RESULTS

Identification of isolates. Of the 253 isolates, 179 (70.8%) and 232 (91.7%) were correctly identified to the species and genus levels, respectively (Table 1). Twenty (7.9%) had scores of <1.700 and were considered to have no identification.

Misidentifications. Using the manufacturer's suggested species cutoff of ≥2.000, all four isolates of *Peptoniphilus indolicus* (according to 16S rRNA gene sequencing) were identified as *Peptoniphilus harei* by MALDI Biotyper analysis. One of the four *Prevotella oralis* isolates was misidentified as *Prevotella nanciencis*. The single *Porphyromonas gulae* isolate was misidentified as *Porphyromonas gingivalis*. Both isolates of *Bacteroides dorei* were misidentified as *Bacteroides vulgatus*, and the single *Bacteroides faecis* isolate was misidentified as *Bacteroides thetaiotaomicron*. Of three isolates of *Actinomyces meyeri*, one was misidentified as *Actinomyces odontolyticus*, and the single *Actinomyces viscosus* isolate was misidentified as *Actinomyces oris*. Using the manufacturer's suggested cutoff values, a single isolate of *Mogibacterium timidum* was misidentified (at the genus level) as *Clostridium halophilum*. *P. indolicus*, *P. oralis*, *P. gulae*, *A. meyeri*, and *A. viscosus* were included in the commercial database, but *B. dorei*, *B. faecis*, and *M. timidum* were not (and were not included in the user-supplemented database).

Analysis using lowered identification score levels. We reanalyzed the data using cutoffs lower than recommended by the manufacturer, as done in prior studies for Gram-positive cocci and *Corynebacterium* species (1, 13). We analyzed percent identifications using species-level cutoffs of ≥1.900, ≥1.800, and ≥1.700 and genus-level cutoffs of ≥1.600, ≥1.500, and ≥1.400 (Table 2). A decrease in the species-level identification cutoff to ≥1.900, ≥1.800, and ≥1.700 produced correct identifications in 204/253 (80.6%), 215/253 (85.0%), and 219/253 (86.6%) isolates, respectively. Lowering the species-level cutoff to ≥1.900 did not change the number of misidentifications compared to that found with the manufacturer's suggested cutoff. Misidentifications increased to 13/253 (5.1%) and 14/253 (5.5%) using species-level cutoff scores of ≥1.800 and ≥1.700, respectively. Lowering of the genus-level cutoffs to ≥1.600, ≥1.500, and ≥1.400 produced correct identifications in 237/253 (93.7%), 243/253 (96.0%), and 245/253 (96.8%) isolates, respectively. Using the same lowered cutoffs, genus-level misidentifications occurred in 1/253 (0.4%), 2/253 (0.8%), and 3/253 (1.2%) isolates, respectively.

DISCUSSION

In our study, the MALDI Biotyper correctly identified 70.8% of anaerobic isolates to the species level and 91.7% to the genus level using the manufacturer-recommended cutoff scores and on-plate formic acid preparation. To our knowledge, this is the first study to utilize an on-plate formic acid preparation for the identification of an entire collection of anaerobic bacterial isolates using the MALDI Biotyper.

Expansion of the system database may yield more overall identifications to the species level. Notably, *B. faecis* and *B. dorei* were not present in the evaluated databases and were likely consequently misidentified as related species with scores of ≥2.000. This illustrates a challenge of using an incomplete database; the user has no easy way to detect such potential misidentifications. Despite representation in the database, *P. oralis*, *P. indolicus*, and *P. gulae*, as well as *A. meyeri* and *A. viscosus*, were misidentified, possibly because of sparse database entries. These organisms do not appear to be closely related to the reported organism based on the 16S rRNA gene sequence.

The MALDI Biotyper was able to correctly identify 91.7% of isolates to the genus level using the manufacturer-suggested cutoffs, with a single misidentification (*M. timidum* misidentified as a *Clostridium* species). Of note, there were no entries for *Mogibacterium* spp. in the supplied manufacturer's library, and the supplemental library contained only single entries for *Mogibacterium neglectum* and *Mogibacterium* species. Previous studies by our group have shown that decreasing cutoffs to ≥1.700 and ≥1.500 for species- and genus-level identification, respectively, allows for correct identification to the species and genus levels for Gram-positive cocci and *Corynebacterium* species. Three additional misidentifications, including two isolates of *P. oralis* misidentified as *P. nanciensis* and the *M. timidum* isolate, which was misidentified as *Clostridium halophilum*, occurred when the species-level cutoff score was lowered to ≥1.700; using a cutoff score of ≥1.700 would otherwise have increased species-level identification to 219/253 (86.6%) of isolates. Lowering cutoffs for genus-level identification to ≥1.600, ≥1.500, and ≥1.400 produced no, one, and two additional misidentifications, respectively. Neither of the two isolates misidentified at the ≥1.400 level were in the databases.

In a previous study by Fedorko et al. (6), anaerobic bacteria identified by the Bruker Biotyper using an off-plate extraction method produced correct species-level identification in 79% of isolates using a cutoff score of ≥2.000 for species-level identification. In our study, 70.8% of isolates were correctly identified using ≥2.000 for a species-level cutoff. Interestingly, all 25 isolates which scored between 1.900 and 1.999 were correctly identified to the species level, an outcome which would result in a comparable identification percentage of 80.6% were cutoff values lowered to ≥1.900. Fedorko and colleagues additionally noted a further increase in species-level identification to 86% when the species-level cutoff score was lowered to ≥1.800 (6). A comparable 85% correct identification was observed at this level in our study. Other studies using the Bruker Biotyper system have shown lower correct species-level identification percentages ranging from 51%, found by Veloo and colleagues (11), to 67.2%, in a study by Justesen and colleagues (7). The importance of the size and quality of the database used in bacterial identification studies by MALDI-TOF MS has been well documented (4, 10, 11, 18) and is likely to have played a role in the differences in identification percentages seen

TABLE 1 Results of MALDI-TOF MS for all study isolates

Anaerobic bacterium tested	Total no. of isolates	No. of isolates:			
		Correctly identified to each level		Not identified	Misidentified (misidentification)
		Genus	Species		
Actinobaculum schaalii	7	7	3	0	0
Actinobaculum urinale	1	0	0	1	0
Actinomyces europaeus	9	8	3	1	0
Actinomyces graevenitzii	10	10	8	0	0
Actinomyces meyeri	3	3	2	0	1 (*Actinomyces odontolyticus*)
Actinomyces neuii	8	6	5	2	0
Actinomyces odontolyticus	9	9	5	0	0
Actinomyces radingae	1	1	1	0	0
Actinomyces turicensis	1	1	1	0	0
Actinomyces urogenitalis	1	1	1	0	0
Actinomyces viscosus	1	1	0	0	1 (*Actinomyces oris*)
Alloscardovia omnicolens	1	1	1	0	0
Anaerostipes caccae	1	0	0	1	0
Atopobium parvulum	3	3	3	0	0
Atopobium rimae	1	1	1	0	0
Bacteroides caccae	2	2	2	0	0
Bacteroides coagulans	1	1	1	0	0
Bacteroides dorei	2	2	0	0	2 (*Bacteroides vulgatus*)
Bacteroides faecis	1	1	0	0	1 (*Bacteroides thetaiotaomicron*)
Bacteroides fragilis	9	9	9	0	0
Bacteroides intestinalis	2	1	0	1	0
Bacteroides ovatus	2	2	1	0	0
Bacteroides pyogenes/denticanum	4	4	3	0	0
Bacteroides salyersiae	1	1	1	0	0
Bacteroides thetaiotaomicron	4	4	4	0	0
Bacteroides ureolyticus	1	1	1	0	0
Bacteroides vulgatus	3	3	3	0	0
Bifidobacterium breve	1	1	1	0	0
Bifidobacterium longum	1	1	1	0	0
Bifidobacterium scardovii	2	2	2	0	0
Blautia coccoides/producta	1	1	1	0	0
Bulleidia extructa	2	2	2	0	0
Butyricimonas virosa	1	1	0	0	0
Campylobacter curvus	1	1	1	0	0
Clostridium butyricum	1	1	1	0	0
Clostridium cadaveris	2	2	2	0	0
Clostridium clostridioforme	1	1	1	0	0
Clostridium difficile	2	2	2	0	0
Clostridium hathewayi	1	1	1	0	0
Clostridium innocuum	2	2	2	0	0
Clostridium paraputrificum	2	2	2	0	0
Clostridium perfringens	6	6	5	0	0
Clostridium ramosum	3	3	3	0	0
Clostridium septicum	3	3	3	0	0
Clostridium symbiosum	1	1	1	0	0
Clostridium tertium	2	2	2	0	0
Clostridium xylanovorans	2	2	2	0	0
Collinsella aerofaciens	1	1	1	0	0
Desulfovibrio fairfieldensis	1	1	1	0	0
Dialister pneumosintes	1	1	1	0	0
Eggerthella lenta	8	8	6	0	0
Finegoldia magna	16	16	12	0	0
Flavonifracter plautii	1	1	1	0	0
Fusobacterium mortiferum	1	1	1	0	0
Fusobacterium necrophorum	3	3	3	0	0
Helcococcus kunzii	1	1	1	0	0
Leptotrichia trevisanii	2	2	2	0	0

(Continued on following page)

TABLE 1 (Continued)

Anaerobic bacterium tested	Total no. of isolates	No. of isolates: Correctly identified to each level: Genus	Species	Not identified	Misidentified (misidentification)
Leptotrichia wadei	1	1	1	0	0
Mobiluncus curtisii	1	1	1	0	0
Mogibacterium timidum	3	0	0	2	1 (*Clostridium halophilum*)
Parvimonas micra	4	4	4	0	0
Parabacteroides goldsteinii	1	1	1	0	0
Peptoniphilus harei	3	3	3	0	0
Peptoniphilus indolicus	4	4	0	0	4 (*Peptoniphilus harei*)
Peptostreptococcus anaerobius	7	6	6	1	0
Porphyromonas asaccharolytica	1	0	0	1	0
Porphyromonas gulae	1	1	0	0	1 (*Porphyromonas gingivalis*)
Porphyromonas levii	1	1	1	0	0
Prevotella bergensis	3	3	2	0	0
Prevotella bivia	9	9	9	0	0
Prevotella buccae	1	1	1	0	0
Prevotella denticola	3	3	3	0	0
Prevotella disiens	1	1	1	0	0
Prevotella intermedia	1	1	1	0	0
Prevotella loescheii	1	0	0	1	0
Prevotella melaninogenica	2	2	2	0	0
Prevotella nigrescens	2	2	2	0	0
Prevotella oralis	4	4	0	0	1 (*Prevotella nanciencis*)
Propionibacterium acnes	9	9	6	0	0
Propionibacterium avidum	3	3	3	0	0
Propionibacterium granulosum	2	2	0	0	0
Propionimicrobium lymphophilum	1	1	0	0	0
Shuttleworthia satelles	2	0	0	2	0
Slackia exigua	2	2	1	0	0
Solobacterium moorei	2	2	2	0	0
Staphylococcus saccharolyticus	10	4	0	6	0
Tissierella praeacuta	1	1	1	0	0
Trueperella bernardiae	1	1	1	0	0
Varibaculum cambriensis	1	0	0	1	0
Veillonella atypica	1	1	1	0	0
Veillonella dispar/parvula	2	2	2	0	0
Veillonella montpellierensis	1	1	1	0	0
Total	253	232	179	20	12 (11 at the species level, 1 at the genus level)
% identification		91.7	70.8	7.9	4.7

in these studies. Fedorko and colleagues utilized an expanded database, which, in addition to the manufacturer's supplied database, included representation for each species of isolate tested (6). As discussed above, our study included isolates which were not represented in either library evaluated and were misidentified at the species or genus level. Additionally, our libraries lacked entries for 8 of the 20 isolates which scored <1.700. The presence of additional representative library entries would likely have de-

TABLE 2 Species- and genus-level identification using manufacturer-recommended and lower cutoff scores

Level and identification	No. of isolates (% of total no. of isolates) in each group with each cutoff: ≥2.000	≥1.900	≥1.800	≥1.700	≥1.600	≥1.500	≥1.400
Species level							
Identified	190 (75.1)	215 (85.0)	228 (90.1)	233 (92.1)			
Misidentified	11 (4.3)	11 (4.3)	13 (5.1)	14 (5.5)			
Correctly identified	179 (70.8)	204 (80.6)	215 (85.0)	219 (86.6)			
Genus level							
Identified				233 (92.1)	238 (94.1)	245 (96.8)	248 (98.0)
Misidentified				1 (0.4)	1 (0.4)	2 (0.8)	3 (1.2)
Correctly identified				232 (91.7)	237 (93.7)	243 (96.0)	245 (96.8)

creased the number of misidentifications and increased the overall correct identification percentage.

Use of an off-plate extraction versus an on-plate formic acid preparation method is unlikely to explain differences in scores between our study and the study by Fedorko et al. (6), although this issue was not formally evaluated. Our group has previously compared off-plate extraction with on-plate preparation methods and shown that there is no statistical difference in identification percentages for yeast and *Corynebacterium* species (16) or for staphylococci, streptococci, and aerobic and facultatively anaerobic Gram-negative bacilli (data not shown).

Utilization of the MALDI Biotyper system provides accurate, rapid, and inexpensive identification of anaerobic bacteria, although use of the manufacturer's cutoff scores resulted in several misidentifications at the species level and a single misidentification at the genus level. Expansion of the library may improve accuracy.

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